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14. ABSTRACT

During the past funding period, we performed TRAIL-TZD-mediated apoptotic studies in androgen dependent (LNCaP and 22RV1) and androgen independent (DU145 and PC3) prostate cancer cells. These revealed that the apoptotic response is maximal in the androgen responsive cells and LNCaP cells produced the best response. Extensive dose course studies revealed that 50μM TZD and 100ng/ml TRAIL combination can produce maximal apoptosis in cells plated at high density. This apoptotic response is also associated with increased β-catenin cleavage, indicating its potential role. To confirm this, β-catenin mutants (D/A) have been created, which will be utilized to confirm whether they represent the caspase resistant β-catenin mutants and to understand their role in apoptosis. TRAIL-TZD studies also revealed that pretreatment with a pharmacological inhibitor of GSK3β (AR-A014418) can sensitize cells potently to TRAIL-induced apoptosis in the absence of TZD. These suggested that inhibition of GSK3β activity might be the major mechanism by which TZD sensitizes prostate cancer cells towards TRAIL. GSK3β might thus represent a novel target for developing prostate cancer therapeutics. The *in vivo* xenograft studies are currently being planned to be performed in nude mice due to a very low tumor take and slow tumor growth in the SCID mice. Once completed these are expected to provide critical information regarding the efficacy of TRAIL-TZD and TRAIL-GSK3β inhibitor combination in regulating prostate tumor growth.

15. SUBJECT TERMS
TRAIL, Troglitazone, beta catenin, GSK3beta, apoptosis

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Table of Contents

Introduction:

Over the last year, we have worked on all the 3 specific aims as was listed in the approved Statement of Work. While significant progress has been achieved in aims 1 and 2, there were setbacks with the animal studies as described under task 3. To resolve these, an amendment to the approved animal protocol (ACORP) has been submitted and approved by Loyola IACUC as well as ACURO. We thus anticipate making significant progress in this area in the next year.

Background:

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer death in males (1), (2). Surgical resection and hormonal therapy (with anti-androgens) are the two major forms of treatment currently available, which is not effective at a late stage (hormone independent forms) of the disease. Designing efficient therapeutic agents that can target both hormone dependent and independent forms of the cancer are thus critically important. Since most of the anticancer therapies limit tumor growth via inducing apoptosis, identification of a novel target for drug induced apoptosis will be helpful for treating resistant forms. One such target is β-catenin, a downstream mediator of Wnt pathway and known to be closely linked with tumorigenesis (3). Overexpression of β-catenin can induce early events of prostate tumorigenesis (4), (5), and contribute to prostate cancer cell growth (6). Activating mutations of β-catenin have also been reported in approximately 5% of human prostate cancers (7), (8). β-catenin can also augment transcriptional function of androgen receptor (AR) (9), (10) (11). Since β-catenin can promote survival via increasing expression of survival related genes (12), (13), decreasing β-catenin expression might be a critical event in activating the apoptotic pathway.

Tumor necrosis factor-related apoptosis- inducing ligand (TRAIL), a member of the tumor necrosis factor super-family of death-inducing ligands, has gained increased attention due to its unique capability of inducing apoptotic cell death specifically in cancer cells, without any significant toxicity towards normal cells (14). TRAIL receptors are expressed in both androgen dependent and independent prostate cancer cells, although, some of these cells develop TRAIL resistance (15). Studies by others have revealed that combinatorial treatment with TRAIL and ligands of PPARγ (Peroxisome Proliferator Activated Receptor gamma) can ameliorate TRAIL resistance and induce apoptosis in TRAIL resistant breast cancer cells (16). Despite this information, the detailed mechanism how this drug combination promotes TRAIL sensitivity is still unknown. Identification of downstream molecules regulating this apoptotic pathway is critically required not only to overcome TRAIL resistance, but also to understand the detailed mechanism involved, which can be utilized towards future drug designing. Since TRAIL receptors are expressed in all prostate cancer cells, this therapy might be effective in targeting prostate cancers irrespective of their androgen status.

In our preliminary data, that supported funding of this grant, co-treatment of TRAIL resistant prostate (LNCaP) and liver (Huh-7) cancer cells with a combination of TRAIL and PPARγ ligand Troglitazone (TZD) reduced TRAIL resistance and significantly increased their apoptotic potential. Interestingly, this apoptosis was also associated with a dramatic reduction in the expression of β-catenin protein and a cleavage of β-catenin preceding combinatorial drug-induced apoptosis. Regulation of β-catenin seemed to be independent of the conventional GSK3β-mediated pathway and involved caspase activation. Based on these, in the current application we proposed to study in detail the role of β-catenin and GSK3β in drug-induced

apoptosis of prostate cancer cells (*in vitro*) and prostate cancer xenografts (*in vivo*). The specific aims included: *1) To determine the role of β-catenin in drug-induced apoptosis of prostate cancer cell lines, 2) To determine the role of GSK3β in potentiating drug-induced β-catenin cleavage and apoptosis and 3) Whether β-catenin mediates drug-induced apoptosis in prostate xenografts in vivo*

Body:

The research accomplishments for the last year along with each task included in the approved Statement of Work are outlined below:

Task 1: To determine the role of β-catenin in drug-induced apoptosis of prostate cancer cell lines (1-36 months)

Subaims 1a-1b:

The results described in the preliminary studies section of this grant proposal were obtained initially utilizing LNCaP prostate cancer cells and Huh-7 hepatocellular carcinoma cells (HCC). After the funding of the grant, extensive studies were designed with TRAIL and Troglitazone (TZD) combination in various prostate cancer cells to determine whether this pathway operates in other prostate cancer cells and is a generalized event. In addition, since LNCaP cells (which respond to this combination treatment with potent apoptosis) are androgen sensitive cells, we also utilized androgen insensitive cell types to determine whether this apoptosis pathway is only specific to androgen sensitive cell types. In order to determine the role of β-catenin in mediating this apoptotic response, the correlation of apoptosis with β-catenin expression and cleavage were also determined. This information is critically important clinically to define which prostate cancer types might respond to this form of therapy. The following results were obtained in the past year:

<u>Effect of TRAIL-TZD combination on the apoptosis potential of androgen sensitive and androgen insensitive prostate cancer cells:</u>

To address this questions we utilized two different cell types that are androgen sensitive (LNCaP and 22RV1) and two that are androgen insensitive (PC3 and DU145). These cells were plated at high density followed by treatment with TRAIL and TZD for 24 hours. Extensive analysis of all these cells showed that the LNCaP and 22RV1 produced maximal response to this combination treatment with a significant increase in PARP cleavage, indicating increased apoptosis (Fig 1). PARP cleavage was much less in the

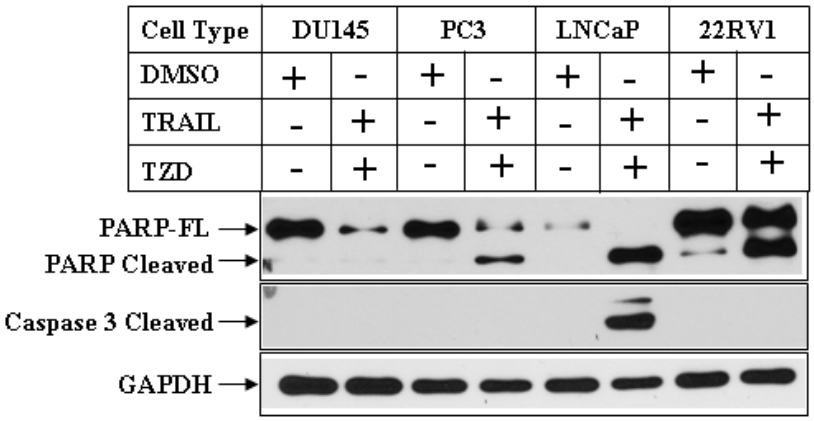

Fig 1: Effect of TRAIL-TZD on prostate cancer cell apoptosis: Androgen sensitive (LNCaP and 22RV1) and androgen insensitive (DU145 and PC3) prostate cancer cells were treated with either DMSO or a combination of 100ng/ml TRAIL and 50μM TZD for 24 hours. Cells were then harvested and equal amount of total cell lysates were analyzed by Western Blots utilizing various antibodies.

PC3 cells and absent in DU145 cells, despite a reduction in the expression of full length PARP. Since these two cell types are androgen insensitive, it is likely that this pathway of apoptosis efficiently targets the androgen sensitive cell types (eg. LNCaP and 22RV1). Cleavage of caspase 3, (the upstream regulator of PARP cleavage in apoptosis), was however, observed only in LNCaP cells, indicating that other caspases might be responsible for PARP cleavage in the 22RV1 and PC3 cells. Since, the apoptotic pathway (indicated by PARP and caspase 3 cleavages) and β-catenin cleavage seemed to be maximum in the LNCaP cells (Fig 1 & Fig 4 below) with this combination treatment, we decided to focus on these cells for the future studies.

Effect of increasing concentrations of TZD and TRAIL on apoptosis of prostate cancer cells:
To determine the optimal concentration of TRAIL and TZD required for inducing maximal apoptosis, Western Blot analyses were performed with LNCaP cell extracts that were treated with increasing concentrations of either TZD or TRAIL. The results indicated that 50-100μM TZD when combined with 100ng/ml TRAIL is optimal for maximal apoptosis, as indicated by PARP and Caspase 3 cleavage (Fig 2A). The results with increasing TRAIL concentrations however, showed a biphasic response as shown in Fig 2B. In these studies, TRAIL at 10-25ng/ml seemed to increase PARP and caspase 3 cleavage significantly, whereas this effect was inhibited at 50-75 ng/ml. At the highest concentration (100ng/ml), the apoptotic response was optimal as indicated by increased PARP and Caspase 3 cleavage. Based on these, the future studies were designed with 50μM TZD and 100ng/ml TRAIL combination.

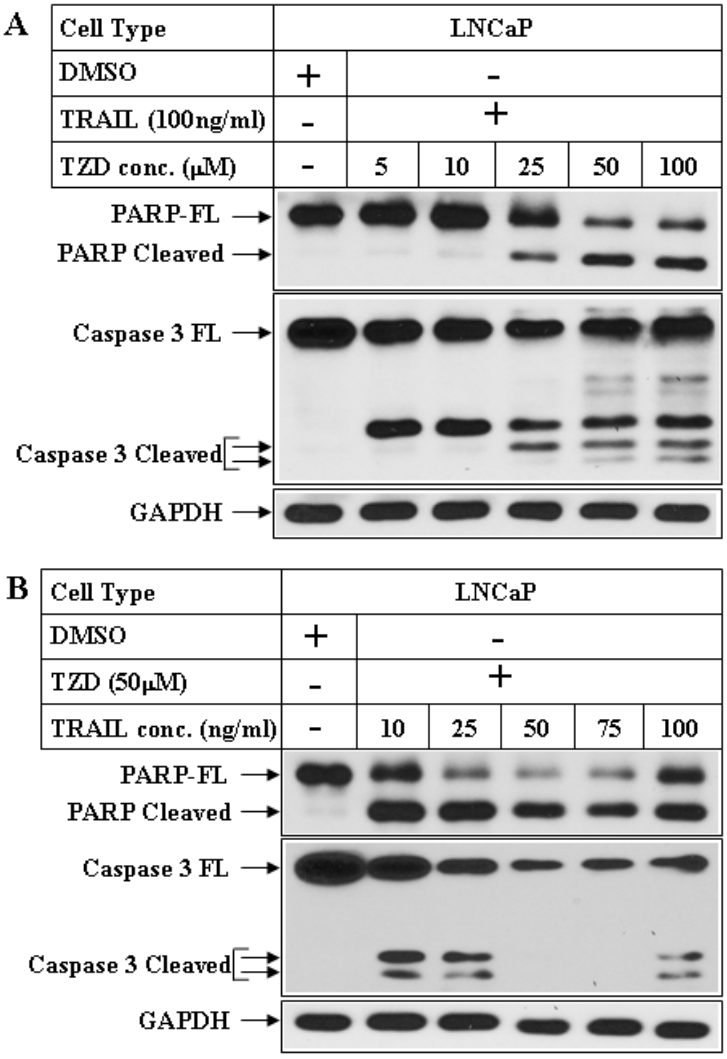

Fig 2: Effect of increasing concentrations of TZD and TRAIL on prostate cancer cell apoptosis: (A) LNCaP cells were treated with DMSO or a combination of 100ng/ml TRAIL and increasing concentrations of TZD (5-100μM). Cells were harvested after 24hours of treatment, followed by Western Blot analysis with the indicated antibodies. ***(B)*** LNCaP cells were treated with either DMSO or a combination of 50 μM TZD and increasing concentrations of TRAIL (10-100ng/ml). Cell lysates were then analyzed by Western Blots as in A.

Effect of Cell density on TRAIL-TZD-induced apoptosis in prostate cancer cells:
While performing these studies, we observed that this pathway of apoptosis is closely related to the density of the cells and the apoptotic potential of the cells were significantly increased with a corresponding increase in cell density. These results are shown in Fig 3 utilizing two different prostate cancer cells LNCaP (A) and DU145 (B). Based on these studies and to obtain maximal apoptosis, these studies in the future will be designed with cells plated at higher density.

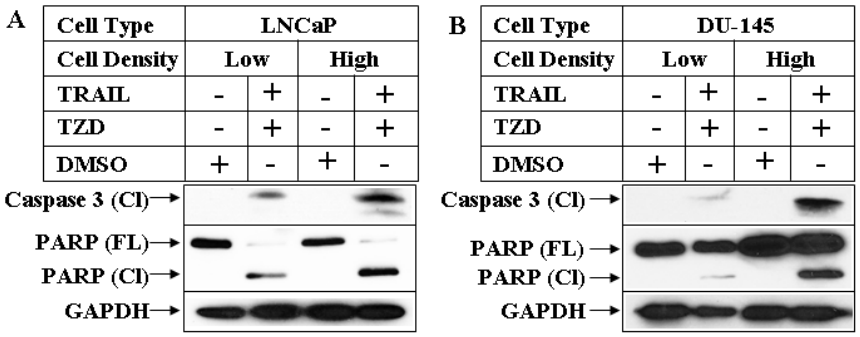

Fig 3: Effect of cell density on TRAIL-TZD-induced apoptosis: (A) LNCaP cells were plated at different densities low (0.3 x 10^6 cells) and high (2.0 x 10^6 cells) and **(B)** DU-145 cells were plated at low (0.1x 10^6) and high (1.0 x 10^6) densities. Both cell types were maintained in serum deficient media for 24 hours and treated with TRAIL-TZD for additional 24hours, followed by Western Blot analysis with the indicated proteins.

Effect of TRAIL-TZD combination on β-catenin levels in androgen sensitive and androgen insensitive prostate cancer cells: As shown in our preliminary results, we observed a close correlation of β-catenin pathway with TRAIL-TZD-induced apoptosis. This was associated with a caspase-induced cleavage and significant decrease in full length β-catenin protein expression in the LNCaP and HCC cells. In order to establish a correlation of β-catenin pathway with prostate cancer cell apoptosis, we designed studies with the four different cell types following treatment with TRAIL and TZD combination.

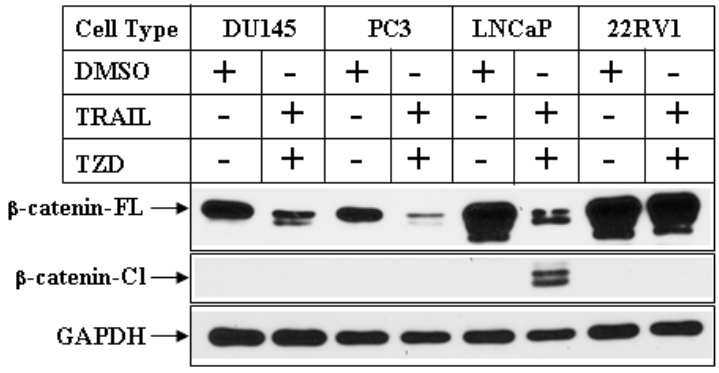

Fig 4: Effect of TRAIL-TZD on β-catenin levels in prostate cancer cells: DU145, PC3, LNCaP and 22RV1 cells were treated as in Fig 1 and analyzed by Western Blots for expression of full length (FL) and cleaved (Cl) β-catenin. The same samples blotted for GAPDH served as controls.

These results showed a distinct cleavage of β-catenin protein in the LNCaP cells when treated with the drug combination (Fig 4, see β-catenin-cl panel). This cleaved form was absent in all

other cell types and was similar to the cleaved caspase 3 expression as shown in Fig 1, suggesting strongly that this cleaved β-catenin expression correlates very well with the apoptotic potential of the prostate cancer cells. Treatment with this drug combination, however, produced a substantial decrease in the expression of the full length protein in almost all the cell types tested (Fig 4, see β-catenin-FL panel). These indicated that the decrease in β-catenin full length protein and β-catenin cleavage operate via two independent pathways with the latter being correlated with apoptosis. It will however, be interesting to determine whether the decrease in full length β-catenin protein is a prerequisite for the induction of apoptosis. In an earlier report, we have demonstrated that PPARγ activation via TZD can lead to a reduction of full length β-catenin expression, which operates via a GSK3β-independent non-conventional pathway (17). It is thus conceivable that this reduction in the full length β-catenin protein expression might be due to the effects of TZD in the drug combination.

Effect of increasing concentrations of TZD and TRAIL on β-catenin expression in prostate cancer cells: In order to determine whether β-catenin cleavage also follows a similar patter as the apoptotic response determined in Figs 2A-B, the cell lysates treated with different concentrations of TZD and TRAIL were also analyzed for changes in β-catenin expression. These results showed a reduction in the level of full length β-catenin with increasing concentrations of TZD and TRAIL (Figs 4A and B). This was also associated with the appearance of the β-catenin cleaved fragment that showed a similar pattern as apoptosis (β-catenin cleaved panel).

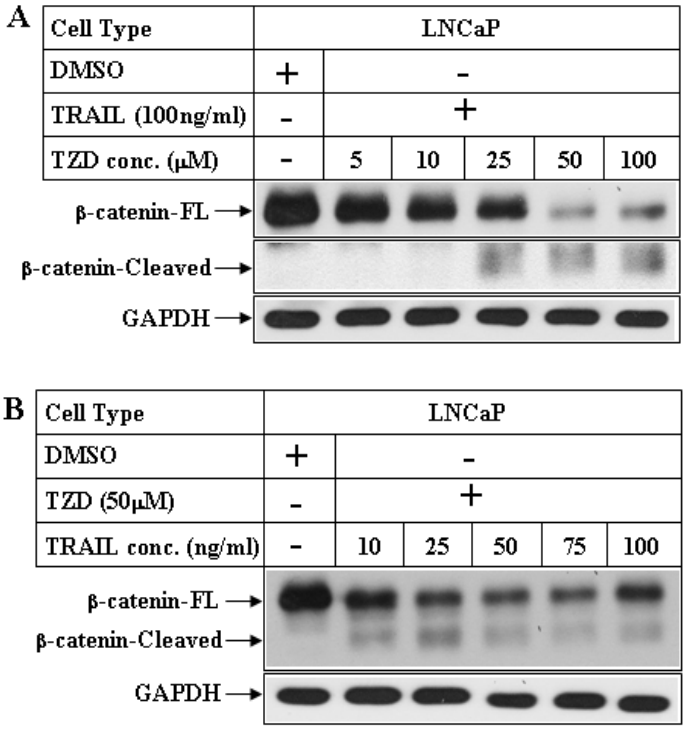

Fig 4: Effect of increasing concentrations of TZD and TRAIL on β-catenin levels in prostate cancer cells: LNCaP cells were treated with ***(A)*** DMSO or a combination of 100ng/ml TRAIL and increasing concentrations of TZD (5-100 μM) or ***(B)*** with either DMSO or a combination of 50μM TZD and increasing concentrations of TRAIL (10-100ng/ml). Cells were harvested after 24hours of treatment, followed by Western Blot analysis with the indicated antibodies.

Subaim 1c:
Based on our earlier results and in order to map the caspase cleavage site on the β-catenin protein, we have also created cleavage resistant mutants of β-catenin which was proposed as part of this subaim. To identify the potential caspase cleavage sites on β-catenin, we used the CASVM Server 1.0 [CASVM: server for SVM prediction of Caspase Substrates Cleavage Sites). In the Western Blot studies, the sizes of the β-catenin cleaved fragments were determined to be ~ 70 KD. In addition, this β-catenin cleaved fragment was detected by an antibody raised against the C-terminal 210 amino acids of β-catenin (BD Biosciences) and not by one raised against the C-terminal 100 amino acids (Zymed). Based on these, we predicted that the cleavage site is between the C-terminal 210 and 100 amino acids. According to the CASVM server, no caspase 3 cleavage sites were detected around that region of β-catenin. However, two potential caspase 8 cleavage motifs [(L/V)EXD] were detected at 583 a.a (LARD) and 624 a.a. (LAQD) of β-catenin protein. Our earlier studies with caspase 8-SiRNA and pretreatment with Caspase 8 inhibitor (IETD-fmk) showed a dramatic reduction in β-catenin cleavage as well as apoptosis when Caspase 8 was inhibited (18), suggesting a predominant role of this caspase in mediating these events. We thus focused on the two potential Caspase 8 cleavage sites on β-catenin to determine their involvement. Accordingly, the following D to A (Aspartate to Alanine) β-catenin mutants were created in pcDNA3 containing myc epitope tag: myc-β-catenin D583A, D624A and D583A/D624A. Studies are currently underway to characterize these mutants and to determine whether these two are the caspase cleavage sites on β-catenin that is targeted during TRAIL-TZD-induced apoptosis.

Task 2: To determine the role of GSK3β in potentiating drug-induced β-catenin cleavage and apoptosis (months 1-24)
Our earlier studies with HCC cells demonstrated that pretreatment with pharmacological inhibitors of the kinase GSK3β potentiates the apoptotic response of TRAIL-TZD combination. Studies in this aim were designed to determine whether GSK3β inhibition contributes towards drug-induced apoptosis in prostate cancer cells and to identify the effective inhibitor combination.

Effect of pharmacological inhibition of GSK3β on TRAIL-TZD-induced apoptosis in prostate cancer cells:
To determine the role of GSK3β in prostate cancer cell apoptosis, we pretreated the LNCaP cells with two different pharmacological inhibitors of GSK3β (AR-A014418 and Kenpaullone) followed by treatment with TRAIL and TZD combination. These results showed that caspase 3 cleavage was significantly higher in the cells pretreated AR-A014418, compared to no pretreatment or Kenpaullone pretreatment (Fig 5). β-catenin cleavage was also showed a similar profile as caspase 3, whereas PARP cleavage was not significantly modulated. In order to determine whether TRAIL-TZD combination resulted in inhibition of GSK3β in the prostate cancer cells, these samples were blotted with an antibody that detects GSK3β when phosphorylated at Ser9 position (inhibitory phosphorylation). These showed a potent increase in $GSK3\beta^{Ser9}$ phosphorylation in all the samples treated with TRAIL-TZD combination (pGSK3β panel). To determine the efficacy of the GSK3β inhibitors, the samples were blotted with a phospho-Glycogen Synthase (pGS) antibody that detects GS (downstream substrate of GSK3β) when phosphorylated by GSK3β.

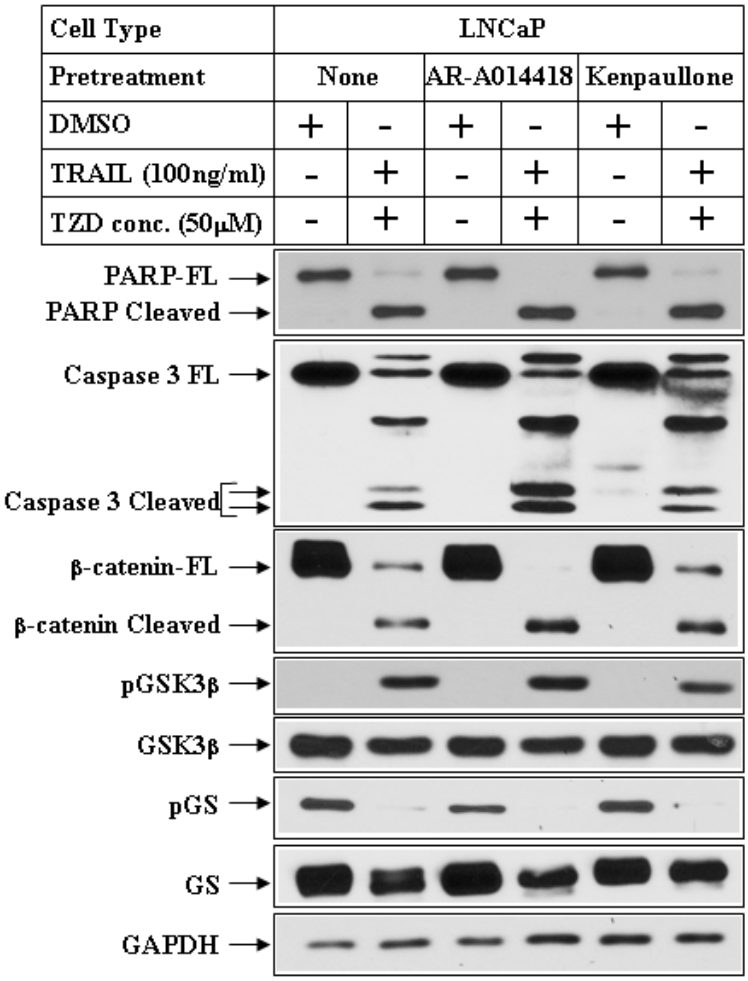

Fig 5: Effect of pharmacological inhibitors of GSK3β on TRAIL-TZD-induced apoptosis in prostate cancer cells: LNCaP cells were pretreated with either none (lanes 1, 2), or 10μM AR-A014418 (lanes 3, 4) or 10μM Kenpaullone (lanes 5, 6) for 1 hour followed by TRAIL+TZD treatment for 24hrs. Western Blot analyses were then perfor med with the antibodies indicated.

As shown in Fig 5 (pGS) panel, TRAIL-TZD treatment decreases pGS levels coinciding with increases in GSK3β phosphorylation (pGSK3β panel), indicating inhibition of the kinase. Pretreatment with AR-A014418 reduced pGS levels in the absenc e of TRAIL-TZD, (compare lane 1 and 3), whereas Kenpaullone was ineffective (lane 5). T hese studies were also perform ed with a higher concentration of AR-A014418 (20μM), which potentiated the apoptotic response even further (data not shown). This suggested that AR-A0144 18 (and not Kenpaullone) effectively inhibited the GSK3β pathway. These interesting results also suggested the possibility that com bining GSK3β inhibitors with TR AIL might effectively elim inate TRAIL resistance and induce apoptosis. In that case it would al so suggest that inhibition of GSK3 β is one of the m ajor mechanisms by which TZD might be sensitizing resistant cancer cells to TRAIL.

Effect of AR-A014418 on TRAIL-induced apoptosis in prostate cancer cells:

To determine whether GSK3β inhibition alone is enough to sens itize the cells to TRAIL-induced apoptosis, the LNCaP cells were pretreated with AR-A014418 followed by treatm ent with TRAIL alone. These studies showed a pot ent increase in PARP, caspase 3 and β-catenin cleavage with AR-A014418 and TR AIL combination, which is equivalent to TRAIL-TZD treatment (Fig 6, com pare lanes 2, 4 and 6). It is thus likely that TZD-m ediated inhibition of GSK3β sensitizes the prostate cancer cells towards TRAIL-induced apoptosis. In fact, cleavage of these proteins (indicati ng apoptosis) was higher with T RAIL + AR-A014418 com bination than TRAIL + TZD. These suggested that TZD sensitizes to TRAIL by antagonizing GSK3 β pathway.

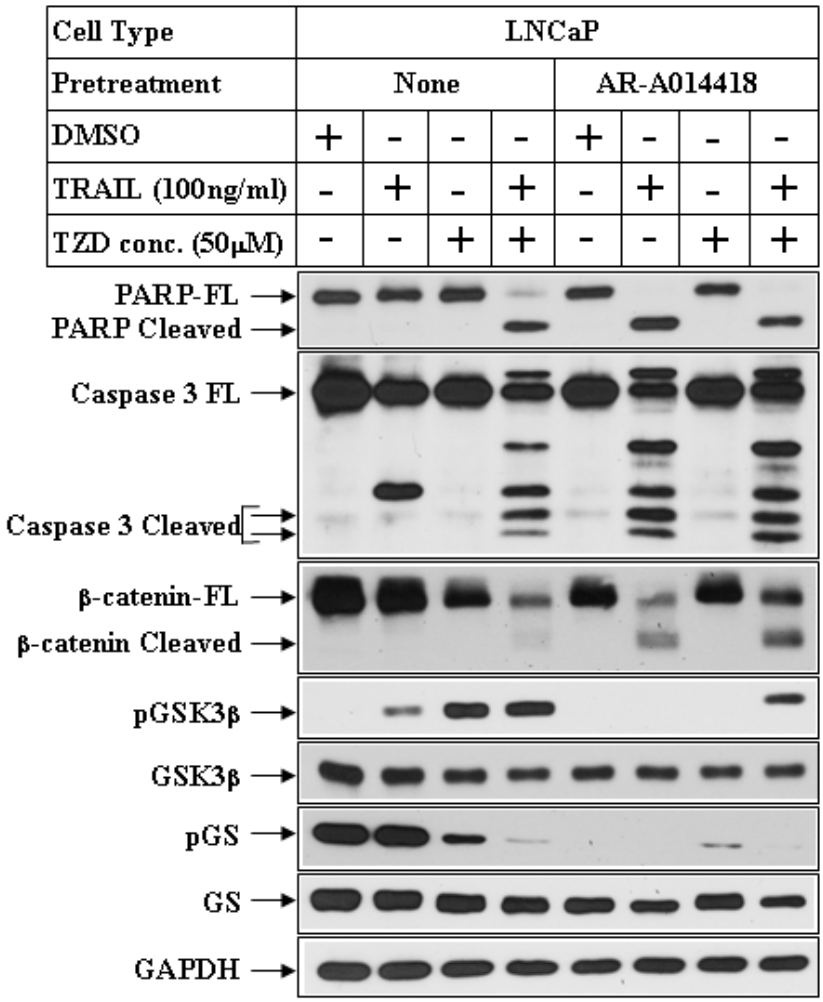

Fig 6: Effect of AR-A014418 on TRAIL-induced apoptosis: LNCaP cells were pretreated w ith none (lanes 1-4) or with 20μM AR-A014418 for 1 hour (lanes 5-8). They were then treated for 24 hours with DMSO or either TRAIL or TZD alone or in combination, followed by Western Blot analysis.

Effect of GSK3β overexpression on TRAIL-TZD-induced apoptosis in prostate cancer cells:

In order to confirm that inhibition of GSK3 β can sensitize prostate cancer cells towards TRAIL-induced apoptosis, HA-tagged GSK3 β-Wild type (WT) and mutant (K/A and S9A) forms were overexpressed in LNCaP cells . The expression of these recombinant GSK3β proteins were first verified in HeLa cells by co transfecting with m yc-tagged β-catenin. As shown in Fig 7, overexpression of both GSK3 β-WT and S9A mutant (phosphorylation deficient and activated

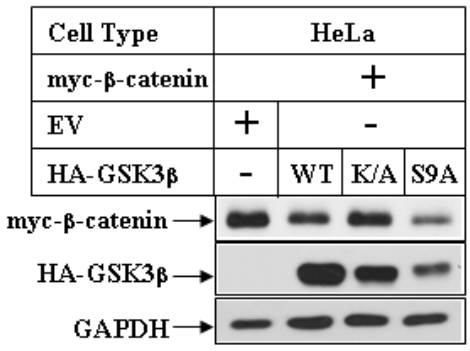

Fig 7: Effect of GSK3β overexpression on β-catenin: HeLa cells were transiently transfected with m yc-β-catenin along with either em pty vector (EV) or HA-tagged GSK3β-WT or mutant vectors. Western Blot analysis was performed with the antibodies shown.

mutant) reduced myc expression whereas the K/A mutant (kinase dead mutant) was unable to regulate ectopic β-catenin expression. This indicated that the GSK3 β constructs expressed the respective proteins correctly.

In the next step, TRAIL-TZD studies were performed with LNCaP cells, following overexpression of either EV or various GSK3β vectors, to determine the effect of GSK3β on this apoptotic pathway. Surprisingly, however, overexpression of both WT and K/A forms seemed to inhibit the apoptosis pathway, whereas the S9A had no effect (Fig 8). Although it is unclear at this point the reason behind the discrepancy between overexpression and AR-A014418 studies, several possibilities might exist: (i) Overexpression of GSK3 β might result in compensatory effect by GSK3α, whereas AR-A014418 might be targeting both isoforms resulting in complete inhibition. (ii) antagonism of apoptosis by GSK3 β might be independent of its kinase activity thus WT and K/A has similar effects and (iii) AR-A014418 targets some other pathways. Future experiments will be designed with siRNA targeting GSK3β and GSK3α to determine whether antagonizing both pathways mimic the effects of AR-A014418 on TRAIL-induced apoptosis.

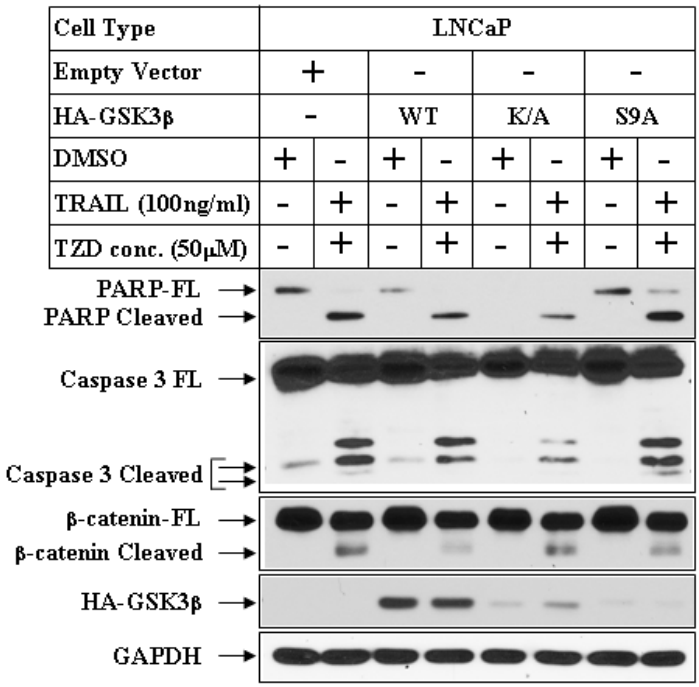

Fig 8: Effect of GSK3β overexpression on TRAIL-TZD-induced apoptosis: LNCaP cells were transiently transfected with either empty vector (lanes 1, 2), or HA-tagged GSK3β WT vector (lanes 3,4), or K/A vector (lanes 5,6) or S9A vectors (7,8). 48hours after transfection they were treated with either DMSO or a combination of TRAIL and TZD, followed by Western Blot analysis.

Task 3: Whether β-catenin mediates drug-induced apoptosis in prostate xenografts *in vivo* (months 1-36)

Studies in this section are expected to address the role of TRAIL-TZD combination as well as β-catenin in mediating apoptosis *in vivo* utilizing a subcutaneous xenograft model. These were initially planned with SCID mice and following approval from Loyola IACUC and ACURO, the xenograft studies were initiated. We injected 5 SCID mice with LNCaP cells (10^6 cells per mice) subcutaneously and observed them for several weeks for tumor development. However, the tumor take was very poor in these mice (only 1 out of 5) and the tumor growth was very slow (> 5 weeks to observe very small palpable tumor). After 8 weeks the tumor was still very small and

not measurable from the skin surface, at which time they were euthanized. This has created a setback in these xenograft studies that were initially planned under this task. To resolve this we have amended our initial animal protocol to perform these xenograft studies in nude mice, which is reported to have better tumor take and xenograft tumor growth. Accordingly, we have received approval from our institutional IACUC for this ACORP amendment, as well as approval from ACURO. New xenograft studies will now be performed in the immediate future as per amended protocol so we can obtain significant progress in this aim in the next year.

Key Research Accomplishments:

1. Identified the prostate cancer cell type that is maximally responsive to the combinatorial treatment with TRAIL and TZD. These also showed that the androgen dependent prostate cancer cells (LNCaP) are the most responsive to this drug combination.
2. Received information regarding the optimal concentration of TRAIL and TZD and the plating cell density at which apoptosis is maximal.
3. Identified the prostate cancer cell type and the optimal drug concentrations that show maximum cleavage of β-catenin cleavage.
4. Created myc-β-catenin mutants (D583A, D624A and D583A/D624A) to map the site of β-catenin cleavage during TRAIL-TZD-induced apoptosis.
5. Determined that pretreatment with a GSK3β inhibitor (AR-A014418) can sensitize the prostate cancer cells towards TRAIL-induced apoptosis in the absence of TZD, suggesting that TZD might be decreasing TRAIL resistance via antagonizing GSK3β.
6. Received IACUC and ACURO approvals for amended ACORP for xenograft studies.

Reportable Outcomes:

None at this time, but once the GSK3β-siRNA studies are completed (in the near future), a manuscript describing these data will be submitted for publication.

Conclusion:

In conclusion, over the past year we have demonstrated utilizing both androgen dependent and independent prostate cancer cells that the androgen dependent cell types (LNCaP cells mostly) respond to this TRAIL-TZD combination treatment via inducing apoptosis. This pathway of apoptosis is always associated with an increased cleavage of β-catenin, and inhibition of β-catenin expression. To understand any potential function of cleaved β-catenin in prostate cancer cell apoptosis, it is necessary to map the cleavage site(s) on β-catenin so that cleavage resistant mutants can be generated. Based on the available database and the approximate size of the cleaved fragments, β-catenin D/A mutants have been generated after mutating two potential caspase 8 cleavage sites. Studies are currently underway to determine whether these are the cleavage resistant mutants, which can then be utilized both *in vitro* and *in vivo* to determine their role on apoptosis. In addition, utilizing a pharmacological inhibitor of GSK3β (AR-A014418), we have demonstrate that the cells can be sensitized to TRAIL-induced apoptosis even in the absence of TZD, suggesting a potential role of GSK3β in apoptotic resistance. More molecular studies are planned to confirm this, which will reveal a novel pathway of ameliorating TRAIL resistance in prostate cancer cells. In addition, the *in vivo* xenograft studies will determine the efficacy of TRAIL-TZD as well as GSK3β inhibitor in controlling prostate tumor growth.

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Appendices:

(See Loyola IACUC approval letter and ACURO approval letter on next page for the ACORP amendment)

LOYOLA
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April 26, 2011

U.S. Army Medical Research and Materiel Command
Animal Care and Use Review Office
504 Scott Street
Fort Detrick, MD 21702-5012
Phone: 301-619-6694
Fax: 301-619-4165
Email: acuro@amedd.army.mil

RE: IACUC APPROVAL
PI: DR. B. RANA; LU#201974 (1.03) Mouse

To Whom It May Concern,

The Loyola University Chicago, Stritch School of Medicine's Institutional Animal Care and Use Committee (JACUC) approved the ACORP; DR. B. RANA; LU#201974 (1.00) Mouse (Beta catenin in prostate cancer apoptosis) on January 06, 2010. The protocol has a full board approval until January 06, 2013.

An amendment, DR. B. RANA; LU#201974 (1.03) Mouse, was approved by Designated Member Review (DMR) on April 07, 2011. The amendment was to change the mouse strain (SCID mice to nude mice).

Loyola University Chicago, Stritch School of Medicine has an Animal Assurance on file with the Public Health Service under A3117-01 approved through 02/28/14 and is a fully AAALAC International accredited institution (certification dated 11/10/10).

If you have any questions or require additional information, please feel free to contact the IACUC Chair, Dr. Jawed Fareed, via the Committee Administrator, telephone (708) 216- 4288; Fax (708) 216-9399.

Sincerely,

Jawed Fareed, Ph.D.
IACUC Chair
Stritch School of Medicine
Loyola University Chicago
Bldg. 120, Suite 313
2160 S. First Avenue
Maywood, IL 60153
Phone (708)216-5581
jfareed@lumc.edu

cc: LU/IACUC File: DR. B. RANA; LU#201974 (1.03) Mouse

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MD 21702-5012

REPLY TO
ATTENTION OF

April 29, 2011

Director, Office of Research Protections
Animal Care and Use Review Office

Subject: Review of USAMRMC Proposal Number PC093099, Award Number W81XWH-10-1-0195 entitled, "Beta Catenin in Prostate Cancer Apoptosis"

Principal Investigator Basabi Rana
Loyola University Chicago
Chicago, IL

Dear Dr. Rana:

Reference: (a) DOD Instruction 3216.01, "Use of Animals in DOD Programs"
(b) US Army Regulation 40-33, "The Care and Use of Laboratory Animals in DOD Programs"
(c) Animal Welfare Regulations (CFR Title 9, Chapter 1, Subchapter A, Parts 1-3)

In accordance with the above references, the amendment to protocol PC093099 entitled, "Beta Catenin in Prostate Cancer Apoptosis," IACUC protocol number 201974 is approved by the USAMRMC Animal Care and Use Review Office (ACURO) for the use of mice and will remain so until its modification, expiration or cancellation. This protocol was approved by the Loyola University Chicago, Stritch School of Medicine IACUC.

When updates or changes occur, documentation of the following actions or events must be forwarded immediately to ACURO:

- IACUC-approved modifications, suspensions, and triennial reviews of the protocol (All amendments or modifications to previously authorized animal studies must be reviewed and approved by the ACURO prior to initiation.)
- USDA annual program/facility inspection reports
- Reports to OLAW involving this protocol regarding

  a. any serious or continuing noncompliance with the PHS Policy;
  b. any serious deviation from the provisions of the Guide for the Care and Use of Laboratory Animals; or
  c. any suspension of this activity by the IACUC

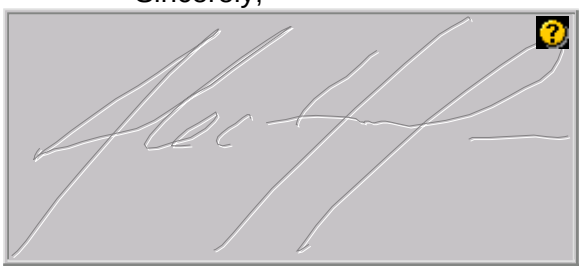
- USDA or OLAW regulatory noncompliance evaluations of the animal facility or program
- AAALAC, International status change (gain or loss of accreditation only)

Throughout the life of the award, the awardee is required to submit animal usage data for inclusion in the DOD Annual Report on Animal Use. Please ensure that the following animal usage information is maintained for submission:

- Species used (must be approved by this office)
- Number of each species used
- USDA Pain Category for all animals used

For further assistance, please contact the Director, Animal Care and Use Review Office at (301) 619-2283, FAX (301) 619-4165, or via e-mail: acuro@amedd.army.mil.

Sincerely,



Alec Hail, DVM, DACLAM
Colonel, US Army
Director, Animal Care and Use
Review Office

Copies Furnished:
Mr. Ayi Ayayi, US Army Medical Research Acquisition Activity (USAMRAA)
Dr. Nrusingha Mishra/MCMR-PLF
Dr. Jawed Fareed, Loyola University Chicago
Ms. Jamie Caldwell, Loyola University Chicago, Stritch School of Medicine